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# Bifunctional Alkylating Agent-Induced p53 and Nonclassical Nuclear Factor $\kappa$ B Responses and Cell Death Are Altered by Caffeic Acid Phenethyl Ester: A Potential Role for Antioxidant/Electrophilic Response-Element Signaling

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## ABSTRACT

Bifunctional alkylating agents (BFA) such as mechlorethamine (nitrogen mustard) and bis-(2-chloroethyl) sulfide (sulfur mustard; SM) covalently modify DNA and protein. The roles of nuclear factor  $\kappa$ B (NF- $\kappa$ B) and p53, transcription factors involved in inflammatory and cell death signaling, were examined in normal human epidermal keratinocytes (NHEK) and immortalized HaCaT keratinocytes, a p53-mutated cell line, to delineate molecular mechanisms of action of BFA. NHEK and HaCaT cells exhibited classical NF- $\kappa$ B signaling as degradation of inhibitor protein of NF- $\kappa$ B ( $I\kappa$ B $\alpha$ ) occurred within 5 min after exposure to tumor necrosis factor- $\alpha$ . However, exposure to BFA induced nonclassical NF- $\kappa$ B signaling as loss of  $I\kappa$ B $\alpha$  was not observed until 2 or 6 h in NHEK or HaCaT cells, respectively. Exposure of an NF- $\kappa$ B reporter gene-expressing HaCaT cell line to 12.5, 50, or 100  $\mu$ M SM activated the reporter gene

within 9 h. Pretreatment with caffeic acid phenethyl ester (CAPE), a known inhibitor of NF- $\kappa$ B signaling, significantly decreased BFA-induced reporter gene activity. A 1.5-h pretreatment or 30-min postexposure treatment with CAPE prevented BFA-induced loss of membrane integrity by 24 h in HaCaT cells but not in NHEK. CAPE disrupted BFA-induced phosphorylation of p53 and p90 ribosomal S6 kinase (p90RSK) in both cell lines. CAPE also increased nuclear factor E2-related factor 2 and decreased aryl hydrocarbon receptor protein expression, both of which are involved in antioxidant/electrophilic response element (ARE/EpRE) signaling. Thus, disruption of p53/p90RSK-mediated NF- $\kappa$ B signaling and activation of ARE/EpRE pathways may be effective strategies to delineate mechanisms of action of BFA-induced inflammation and cell death signaling in immortalized versus normal skin systems.

Bifunctional alkylating agents (BFA) chemically modify DNA and other macromolecules with which they react (Papirmeister et al., 1991). The BFA mechlorethamine (2-chloro-*N*-(2-chloroethyl)-*N*-methyl-ethanamine, nitrogen mustard, NM) is a topical chemotherapeutic agent typically used to treat cutaneous T-cell lymphomas (de Quatrebarbes et al., 2005). A regimen

involves topical application of a 0.02% solution ( $\sim$ 1 mM) (de Quatrebarbes et al., 2005). Occupational exposure to the BFA bis-(2-chloroethyl) sulfide (sulfur mustard, SM) (Davis and Aspera, 2001) has been a concern because SM has known mutagenic properties and can cause significant skin blistering (Papirmeister et al., 1991). No completely efficacious chemoprotectant/therapeutic exists that ameliorates BFA-induced cutaneous intolerance, inflammation, blister formation, cancerous and precancerous lesions, and cell death.

The nuclear factor  $\kappa$ B (NF- $\kappa$ B)/Rel family of transcription factors is involved in the expression of genes related to growth, differentiation, development, and inflammation (Chen and Green, 2004). However, less is known about the

The opinions or assertions contained herein are the private views of the authors and are not to be construed as official or as reflecting the views of the Department of the Army or the Department of Defense.

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**ABBREVIATIONS:** BFA, bifunctional alkylating agent(s); NM, nitrogen mustard (bis-(2-chloroethyl) methylamine or mechlorethamine); SM, sulfur mustard (bis-(2-chloroethyl) sulfide); NF- $\kappa$ B, nuclear factor  $\kappa$ B; TNF $\alpha$ , tumor necrosis factor- $\alpha$ ;  $I\kappa$ B, inhibitor protein of nuclear factor  $\kappa$ B; IKK,  $I\kappa$ B kinase(s); IL, interleukin; ATM, ataxia-telangiectasia-mutated; ATR, ataxia-telangiectasia-related; ARE/EpRE, antioxidant response element/electrophilic response element; CAPE, caffeic acid phenethyl ester; NHEK, normal human epidermal keratinocytes(s); CEES, 2-chloroethyl ethylsulfide; TDG, thiodiglycol (2,2-thiodiethanol); DMSO, dimethyl sulfoxide; HU, hydroxyurea; LDH, lactate dehydrogenase; AhR, aryl hydrocarbon receptor; p90RSK, p90 ribosomal S6 kinase; Nrf2, nuclear factor E2-related factor 2; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal-activated kinase; XRE, xenobiotic response element(s); IB, immunoblotted; PAGE, polyacrylamide gel electrophoresis.



apparent role of NF- $\kappa$ B in promoting cell death. The tumor suppressor protein p53 is a vital mediator of apoptosis following exposure to DNA-damaging agents (Kohn and Pommier, 2005). It has become clear that both NF- $\kappa$ B and p53 can be activated by similar stimuli/insults. For example, DNA-damaging agents induce a "nonclassical" NF- $\kappa$ B pathway (Boland et al., 1997; Bohuslav et al., 2004), and NF- $\kappa$ B mediates cell death signaling following exposure to DNA-damaging agents (Kasibhatla et al., 1998). Reactive oxygen species, known activators of NF- $\kappa$ B, are also potent activators of p53. Oxidative stress may play a significant role in p53 activation following exposure to chemotherapeutics (Martindale and Holbrook, 2002). Furthermore, functional NF- $\kappa$ B and p53 activities exhibit mutual cross-talk whereby the levels and activation of one modulate the activation of the other. The balance of activity and cross-talk between NF- $\kappa$ B and p53 pathways influences the final outcome of cell survival or death (Webster and Perkins, 1999; Fujioka et al., 2004). Delineating the cell signaling responses of these important transcription factors may contribute to understanding the etiology and ameliorating the side effects of exposure to BFA.

Whereas nonclassical NF- $\kappa$ B activity induced by DNA-damaging agents is incompletely understood, the "classical" molecular events that lead to the activation of NF- $\kappa$ B, such as those induced by tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), are relatively well understood (Chen and Greene, 2004). In this classical NF- $\kappa$ B pathway, exposure to TNF $\alpha$  activates inhibitor protein of NF- $\kappa$ B (I $\kappa$ B) kinases (IKK) that phosphorylate I $\kappa$ B $\alpha$ , the inhibitor protein of NF- $\kappa$ B. Phosphorylation of I $\kappa$ B $\alpha$  results in its degradation, which allows NF- $\kappa$ B to translocate into the nucleus. NF- $\kappa$ B then binds to  $\kappa$ B response elements to activate expression of NF- $\kappa$ B-responsive inflammatory mediators, such as interleukin (IL)-8 and/or IL-6 (Zhang et al., 1994; Ritchie et al., 2004). Although exposure to BFA induces accumulation of IL-8 and IL-6 (Dillman et al., 2004), it is thought that DNA-damaging events result in activation of a nonclassical NF- $\kappa$ B pathway that does not rely on IKK-mediated phosphorylation of I $\kappa$ B $\alpha$  and subsequent degradation (Bohuslav et al., 2004; Ryan et al., 2004). The precise mechanisms by which BFA activate this nonclassical NF- $\kappa$ B signaling pathway remain to be delineated.

The tumor suppressor p53 plays a pivotal role in activating and integrating cellular responses to a wide range of environmental stressors, including DNA-damaging agents (Lowe et al., 1993; Rosenthal et al., 1998; Kohn and Pommier, 2005). p53 coordinates a shift in gene expression to promote growth arrest or cell death genes and also blocks the expression of genes that stimulate growth or block cell death. Similar to NF- $\kappa$ B, p53 will not become an active transcription factor until it is dissociated from its inhibitory protein, MDM2. DNA-damaging events activate kinases such as ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia-related (ATR) that phosphorylate p53 and/or MDM2, resulting in dissociation of these proteins and translocation of p53 into the nucleus. DNA damage-initiated phosphorylation of p53 at serine 15 contributes to dissociation from MDM2 and correlates with a p53 response (Shieh et al., 1997; Kohn and Pommier, 2005). Whereas BFA initiate a p53 response, BFA-induced cross-talk between p53 and NF- $\kappa$ B signaling remains to be elucidated.

The precise mechanisms that govern the NF- $\kappa$ B and p53

responses induced by BFA are not completely understood. Here, we delineate the initial responses of the NF- $\kappa$ B and p53 signaling pathways, as well as the antioxidant-response element/electrophilic response element (ARE/EpRE) pathway to BFA-induced insult. Normal and immortalized human epidermal keratinocytes and the phenolic compound caffeic acid phenethyl ester [phenethyl (*E*)-3-(3,4-dihydroxyphenyl)prop-2-enoate, CAPE], a potent inhibitor of NF- $\kappa$ B activity, were utilized to delineate molecular mechanisms of BFA-induced signaling.

## Materials and Methods

**Cell Culture.** Normal human epidermal keratinocytes (NHEK) from breast skin were obtained as cryopreserved first passage stocks from Cascade Biologics (Eugene, OR). Cells were seeded at  $1.9 \times 10^5$  cells into 75-cm<sup>2</sup> flasks. Cells were grown in serum-free supplemented keratinocyte growth medium (EpiLife, Cascade Biologics) to 70 to 80% density before trypsin detachment and reseeding at  $2.4 \times 10^4$  cells/well in six-well plates or  $2.0 \times 10^3$  cells/well in 24-well plates. Second through fifth passage plates of NHEK at 90 to 95% confluence were used for exposures. The NHEK cells were grown at 37°C with 5% CO<sub>2</sub>.

HaCaT cells (Boukamp et al., 1988), a p53-mutated keratinocyte cell line, were seeded at  $1.9 \times 10^5$  cells into 75-cm<sup>2</sup> flasks or  $2.4 \times 10^4$  cells/well in six-well plates or  $2.0 \times 10^3$  cells/well in 24-well plates. Cells were grown in minimal essential medium growth medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum to 70 to 80% density before trypsin detachment and reseeding at  $2.4 \times 10^4$  cells/well in six-well plates or  $2.0 \times 10^3$  cells/well in 24-well plates. HaCaT cells at 90 to 95% confluence were used for exposures. The HaCaT cells were grown at 37°C with 5% CO<sub>2</sub>.

**Stable Keratinocyte Transfection.** The ability of alkylating agents to activate NF- $\kappa$ B was monitored using an NF- $\kappa$ B response element-driven luciferase reporter gene. The reporter plasmid pNF $\kappa$ B-TA-Luc (BD Biosciences, San Jose, CA) contained four tandem copies of the NF- $\kappa$ B consensus sequence (GGGAATTTCC). GenePorter 2 reagent with diluent B (Gene Therapy Systems, Inc., San Diego, CA) was used to cotransfect 1.67  $\mu$ g of pNF $\kappa$ B-TA-Luc and 0.33  $\mu$ g of pPUR (to allow clonal selection) (BD Biosciences) into HaCaT cells in six-well plates, according to recommended procedures. Stable transfectants were selected in medium containing 1.0  $\mu$ g/ml puromycin (Sigma-Aldrich) for 14 days. Isolated colonies were expanded and analyzed for luciferase inducibility by human TNF $\alpha$  (R&D Systems, Inc., Minneapolis, MN). The subclone designated here as HaCaT.AL (Altered Line) was used for these studies based on its robust inducibility by TNF $\alpha$  and relatively low uninduced levels of luciferase activity.

**Chemicals and Exposures.** NM, 2-chloroethyl ethylsulfide (CEES), and 2,2-thiodiethanol [thiodiglycol (TDG)], each purchased from Sigma-Aldrich, were prepared as 4 mM stock solutions in dimethyl sulfoxide (DMSO). These stock solutions were placed on ice and immediately diluted into media for cell exposures. A 20 mM hydroxyurea (HU; Sigma-Aldrich) solution was made directly in cell media and added to the cells. A frozen aliquot of neat SM in keratinocyte growth medium was thawed and vortexed to generate a 4 mM SM stock solution. This stock solution was placed on ice and immediately diluted into media to expose cells to SM. For pharmacological treatment experiments, CAPE (Alexis Platform Axxora, LLC, San Diego, CA) was dissolved in DMSO (30 mg/ml = 105 mM stock). These stock solutions were placed on ice and immediately diluted into media (1:1000; 30  $\mu$ g/ml = 105  $\mu$ M) to treat cells before or after the addition of alkylating agent. Cells were maintained at 37°C with 5% CO<sub>2</sub> during pretreatments, left at 37°C with room CO<sub>2</sub> concentrations during alkylating agent exposures for a maximum of 30 min, and returned to 37°C with 5% CO<sub>2</sub> for the remainder of the postexposure time period.

**Lactate Dehydrogenase Activity Assay.** Lactate dehydrogenase (LDH) activity in cell medium was measured using the Cyto-



Tox-ONE Homogenous Membrane Integrity Assay Kit (Promega, Madison, WI). LDH activity is an indicator of cell membrane integrity and was measured as a correlate of cell viability (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). At the indicated time point, cell growth medium was transferred to a 96-well, black plate and mixed with an equal volume of assay buffer. After 10 min of incubation at room temperature, stop solution was added to each well. Fluorescence was recorded with a Genios (TECAN US, Research Triangle Park, NC) plate reader with an excitation wavelength filter for 530 nm and an emission filter for 580 nm. The cells remaining from each experiment were then lysed for total LDH associated with intact cells, protein analyses by immunoblotting, or reporter gene assays.

**Isolation of Proteins and Gel Electrophoresis.** The culture media were removed from the exposed cells, and the cells were washed with Hanks' balanced salt solution (Sigma-Aldrich). The cells were lysed in 250  $\mu$ l of 4 $\times$  SDS sample buffer (250 mM Tris, pH 6.8, 20% glycerol, 8% SDS, 400 mM dithiothreitol, and 0.01% bromophenol blue) and scraped from the plates. Lysates were triturated with 25-gauge, 5/8 needles using 1-ml syringes. Protein was fractionated on 7.5 or 12.5% Criterion Precast SDS polyacrylamide gels (Bio-Rad, Hercules, CA).

**Immunoblotting.** Proteins normalized to total plated cell number were transferred from polyacrylamide gels to polyvinylidene fluoride membrane (Hybond; GE Healthcare Life Sciences, Piscataway, NJ) by electroblotting. The membranes were blocked with 5% bovine serum albumin in Tris-buffered saline/Tween 20 (10 mM Tris, pH 7.5, 100 mM sodium chloride, and 0.1% Tween 20) and probed using the rabbit polyclonal phospho-p53 (Ser15) antibody (catalog no. 9284; Cell Signaling Technology, Beverly, MA), the rabbit polyclonal p53 antibody (catalog no. 9282; Cell Signaling Technology), the rabbit polyclonal I $\kappa$ B $\alpha$  antibody (catalog no. 9242; Cell Signaling Technology), the rabbit polyclonal anti-phospho p90 ribosomal S6 kinase (p90RSK) (S380) (catalog no. 9341; Cell Signaling Technology), the rabbit polyclonal anti-p90RSK (catalog no. 9347; Cell Signaling Technology), the rabbit polyclonal anti-nuclear factor E2-related factor 2 (Nrf2) (H300) antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or the rabbit polyclonal anti-aryl hydrocarbon receptor (AhR) antibody (catalog no. SA210; Biomol International, Plymouth Meeting, PA). Primary antibodies were diluted in 5% bovine serum albumin in Tris-buffered saline/Tween 20. Primary antibody was detected via an alkaline phosphatase conjugated mouse anti-rabbit secondary antibody (Zymed Laboratories, Invitrogen Immunodetection, Carlsbad, CA) and enhanced chemifluorescence (GE Healthcare Life Sciences). The fluorescent signal was detected and visualized using a Storm 860 scanner (Molecular Dynamics, Sunnyvale, CA) and analyzed using ImageQuant software (Molecular Dynamics). To strip membranes of antibody when necessary, a glycine buffer (pH 2.2) was used followed by blocking and reprobing with the appropriate antibody.

**Luciferase Reporter Gene Activity Assay.** The use of a long-lasting luminescent substrate (Steady-Glo; Promega) provides a stable luminescent signal ( $t_{1/2}$  ~5 h) and enables cell lysis and luciferase activation directly in the culture medium or lysis buffer. HaCaT.AL cells, plated and exposed in triplicate as indicated above, were washed with phosphate-buffered saline and lysed with Glo Lysis Buffer (Promega). Plates were sonicated briefly on ice to enhance cell disruption. Equal volumes of each sample were transferred to a 96-well, black, flat-bottom plate (USA Scientific, Ocala, FL), mixed with Steady-Glo Luciferase Assay reagent (Promega), and incubated at room temperature for 5 min. Luminescence was recorded using a Genios (TECAN) plate reader. The data were plotted as normalized luciferase activity, corrected for vehicle control luciferase activity, and normalized to LDH activity data of treatment/exposure group.

**Statistical Analyses.** Normalized luciferase activity and the percent loss of membrane integrity were expressed as the average  $\pm$  S.E.M. ( $n = 3$ ) and were analyzed for statistical significance using

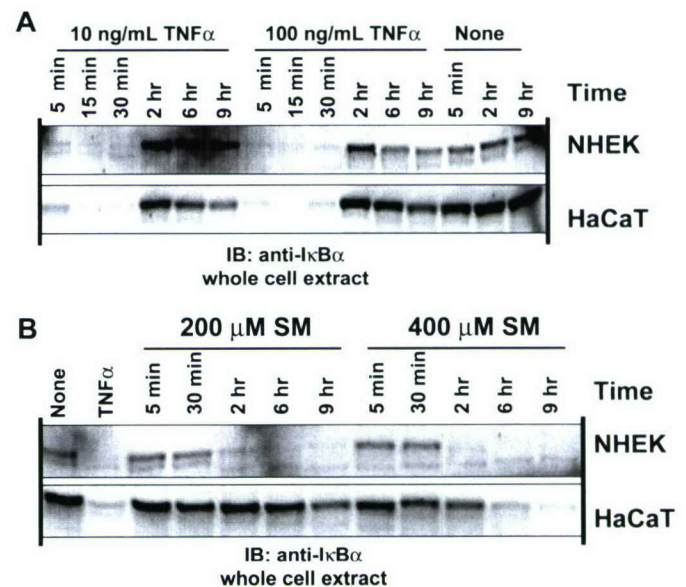
Bonferroni's adjusted  $t$  test to control the experiment-wise error rate at 5%.

**Microscopy.** Digital image photographing was performed using an Olympus CKX41 Culture Microscope (WHB10 $\times$  eyepiece with a 10 $\times$  objective) and DP12 Microscope Digital Camera System (Olympus America, Inc., Melville, NY). Images were compiled or digitally magnified using Adobe Photoshop (Adobe Systems, Mountain View, CA).

## Results

**Alkylating Agents Induce Nonclassical NF- $\kappa$ B Signaling.** We compared the classical NF- $\kappa$ B signaling mediated by TNF $\alpha$  with alkylating agent-induced NF- $\kappa$ B signaling by monitoring I $\kappa$ B $\alpha$  protein expression. NHEK and HaCaT cells have similar responses when exposed to TNF $\alpha$  (Fig. 1A, TNF $\alpha$ ). Exposure of either cell line to 10 or 100 ng/ml of TNF $\alpha$  for even 5 min results in loss of I $\kappa$ B $\alpha$  compared with the untreated cells (Fig. 1A, None). I $\kappa$ B $\alpha$  levels are restored 2 h after TNF $\alpha$  exposure. These data suggest that both NHEK and HaCaT cells exhibit classical NF- $\kappa$ B signaling mechanisms.

NF- $\kappa$ B signaling pathways have been considered a potential pharmacological target for cotreatment with NM-related therapeutics (Sanda et al., 2005) or for development of therapeutics for exposure to SM (Atkins et al., 2000). Therefore, we examined whether alkylating agents induce classical NF- $\kappa$ B signaling in keratinocytes. Interestingly, when exposed to alkylating agents, keratinocytes do not exhibit the classical TNF $\alpha$ -like response (Fig. 1B, denoted by rapid I $\kappa$ B $\alpha$



**Fig. 1.** BFA induce nonclassical NF- $\kappa$ B signaling. A, immortalized HaCaT keratinocytes and NHEK were exposed to media alone (none), 10 ng/ml TNF $\alpha$ , or 100 ng/ml TNF $\alpha$  for 5 min, 15 min, 30 min, 2 h, 6 h, or 9 h. Whole-cell extracts were isolated and immunoblotted (IB) using specific anti-I $\kappa$ B $\alpha$  antibodies following separation by SDS-polyacrylamide gel electrophoresis (PAGE). I $\kappa$ B $\alpha$  levels decreased ~4-fold 5 min after exposure to 10 ng/ml TNF $\alpha$  (compared with 5 min time-matched control) and are restored at 2 h in both NHEK and HaCaT cells. B, HaCaT and NHEK cells were exposed to media (none), 100 ng/ml TNF $\alpha$  for 5 min or 200 or 400  $\mu$ M SM for 5 min, 30 min, 2 h, 6 h, or 9 h. Whole-cell extracts were isolated and IB using specific anti-I $\kappa$ B $\alpha$  antibodies following separation by SDS-PAGE. In NHEK exposed to 200  $\mu$ M SM, I $\kappa$ B $\alpha$  levels decreased <2-fold through 2 h, ~3-fold at 6 h, and ~4-fold at 9 h. In NHEK exposed to 400  $\mu$ M SM, I $\kappa$ B $\alpha$  levels decreased <2-fold through 30 min, ~3-fold at 2 h, ~5-fold at 6 h, and ~4-fold at 9 h. In HaCaT cells exposed to 200  $\mu$ M SM, I $\kappa$ B $\alpha$  levels decreased <2-fold through 6 h and ~2-fold at 9 h. In HaCaT cells exposed to 400  $\mu$ M SM, I $\kappa$ B $\alpha$  levels decreased <2-fold through 2 h, ~4-fold at 6 h, and ~8-fold at 9 h.



degradation as in A). In NHEK, observable  $I\kappa B\alpha$  loss occurs by 2 h following exposure to SM. Concentrations of SM less than 100  $\mu M$  do not result in observable loss of  $I\kappa B\alpha$  within 4 h of exposure (not shown). HaCaT cells exposed to 200  $\mu M$  SM show loss of  $I\kappa B\alpha$  by 9 h after exposure (Fig. 1B). Exposure to 400  $\mu M$  SM did not result in the immediate degradation of  $I\kappa B\alpha$  in either cell type. Furthermore, exposure to 200  $\mu M$  BFA SM or NM, but not the monofunctional CEES or TDG, resulted in decreased levels of  $I\kappa B\alpha$  by 4 h (not shown). These results support recent findings that DNA-damaging agents can induce a delayed, nonclassical NF- $\kappa B$  signaling pathway (Bohuslav et al., 2004).

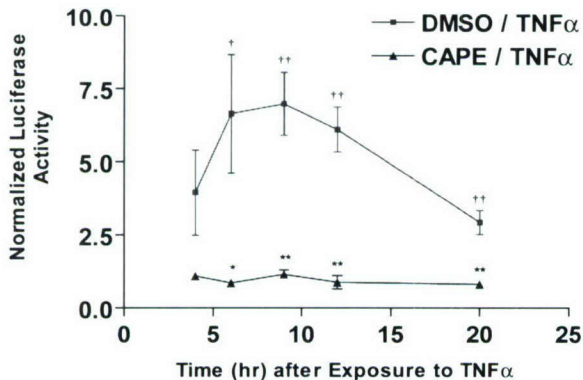
**CAPE Inhibits Classical NF- $\kappa B$ -Mediated Reporter Gene Activity.** CAPE, a potent inhibitor of NF- $\kappa B$  activity, is considered a nontraditional inhibitor because it does not prevent  $I\kappa B\alpha$  degradation (Natarajan et al., 1996). To verify whether CAPE could inhibit either the classical TNF $\alpha$ -mediated or the nonclassical alkylating agent-mediated NF- $\kappa B$  activity, keratinocytes were stably transfected with an NF- $\kappa B$  reporter construct containing multiple  $\kappa B$ -consensus elements to drive expression of a luciferase reporter gene. Exposure of the cells to a saturating concentration of 100 ng/ml of TNF $\alpha$  resulted in a 30-fold increase in detected luciferase activity by 6 h after exposure (not shown). Cells pretreated with vehicle (DMSO) and then exposed to 0.5 ng/ml of TNF $\alpha$  exhibited an  $\sim 6$ -fold increase in luciferase activity between 6 and 12 h after exposure (Fig. 2, DMSO/TNF $\alpha$ ). There was, however, significant inhibition of TNF $\alpha$ -mediated luciferase activity after cells were pretreated with CAPE (Fig. 2, CAPE/TNF $\alpha$ ). These data show that CAPE is a potent inhibitor of classical NF- $\kappa B$  activity in keratinocytes.

**CAPE Inhibits Nonclassical NF- $\kappa B$ -Mediated Reporter Gene Activity.** CAPE was utilized in an attempt to inhibit alkylating agent-induced nonclassical NF- $\kappa B$ -mediated cell signaling. The reporter gene keratinocyte cell line was exposed to increasing concentrations of SM (Fig. 3A). Exposure to 12.5, 50,

or 100  $\mu M$  SM resulted in a 50 to 75% increase in luciferase activity by 9 h after exposure compared with the untreated control cell luciferase activity level (i.e., the value of 1). Luciferase activity returned to untreated control levels 20 h after the initial SM insult. Exposure to 200  $\mu M$  SM decreased the level of luciferase activity to levels  $\sim 40\%$  less than untreated control activity by 4 h after exposure. By 12 h after exposure to 200  $\mu M$  SM, luciferase activity increased by 40 to 50%, but only back to untreated control levels (i.e., 1). Exposure to NM resulted in a similar response as compared with SM exposure, whereas exposure to CEES, even up to an 800  $\mu M$  concentration, resulted in no increase in luciferase activity (not shown). Reporter gene-containing keratinocytes were also pretreated with 10 (no change, not shown) or 105  $\mu M$  CAPE for 1.5 h before exposure to 12.5 (Fig. 3B), 50 (Fig. 3C), 100 (Fig. 3D), or 200  $\mu M$  (Fig. 3E) alkylating agent. CAPE significantly decreased alkylating agent-induced NF- $\kappa B$  activation (Fig. 3, B–E). CAPE maintains NF- $\kappa B$  activity at or below background levels even after exposure to alkylating agents.

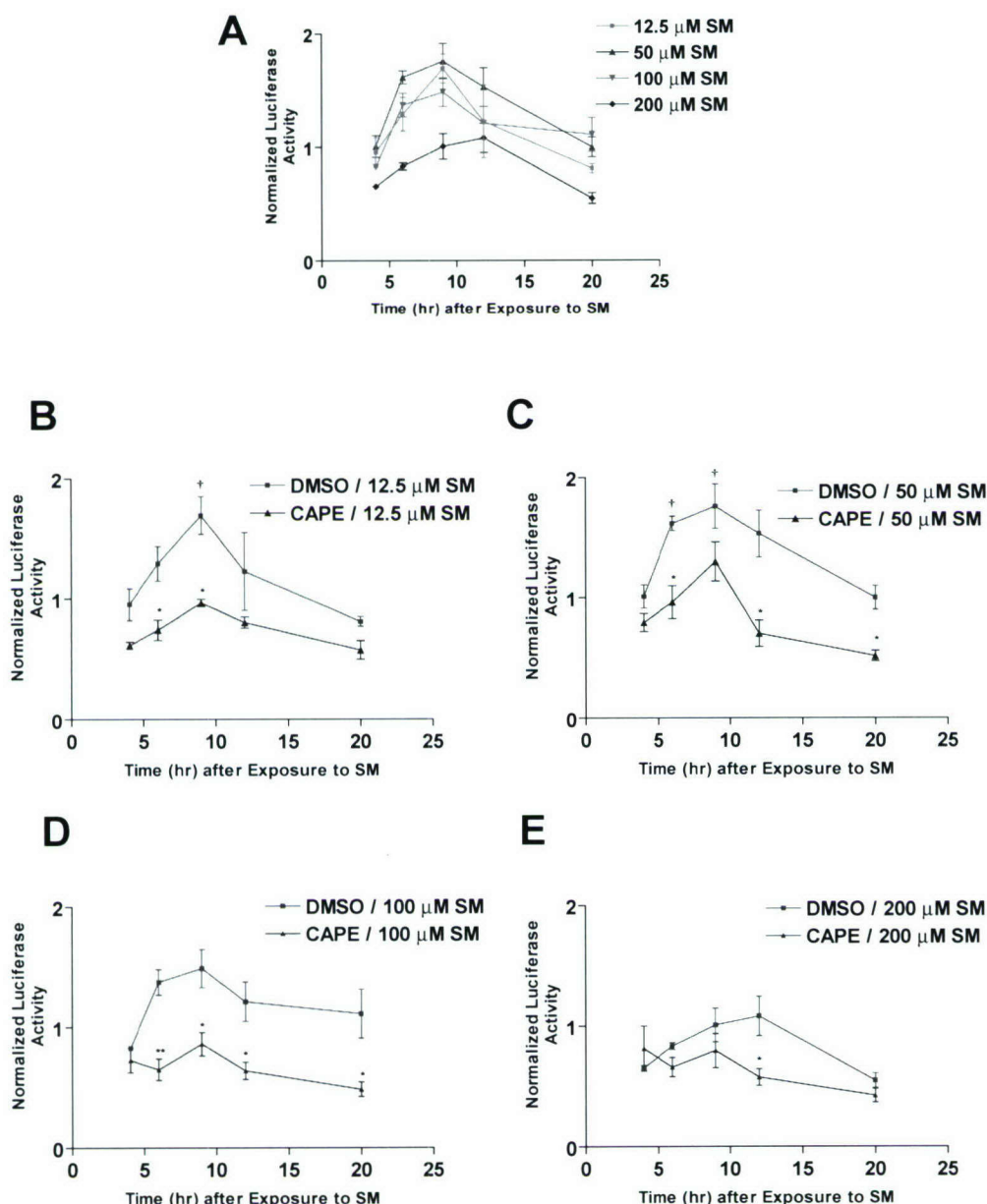
**CAPE Decreases Alkylating Agent-Induced Loss of Membrane Integrity.** Keratinocytes exposed to BFA undergo cell death via an apoptotic-necrotic continuum (Ray et al., 2005). CAPE itself is a known radiosensitizer (Lin et al., 2006) and inducer of apoptosis in a variety of cell lines (Chiao et al., 1995). We examined whether the combination of CAPE and alkylating agent would potentiate loss of membrane integrity and cell death, or whether interfering with NF- $\kappa B$  signaling, which is involved in both proapoptotic and anti-apoptotic mechanisms, would lead to cell survival. NHEK or HaCaT keratinocytes were either pretreated for 1.5 h with CAPE before exposure to alkylating agent for 24 h or first exposed to alkylating agent and post-treated 30 min later with CAPE. LDH activity was assayed in culture media as a measure of cell membrane integrity, a correlate of cell viability. We observed that CAPE alone had no effect on HaCaT keratinocytes (Fig. 4A, Cont) but resulted in an additional  $\sim 60\%$  loss of membrane integrity in NHEK compared with vehicle-treated control cells (Fig. 4B, Cont). In addition, in HaCaT cells, CAPE significantly decreased the SM- and NM-induced loss of membrane integrity out to 24 h (Fig. 4A, Pre: Vehicle versus Pre: CAPE). In NHEK, CAPE did not prevent SM-induced loss of membrane integrity but moderately reduced NM-induced LDH activity (Fig. 4B, Pre: Vehicle versus Pre: CAPE). No potentiating effect of CAPE in combination with BFA was observed. Interestingly, virtually identical responses were observed for HaCaT or NHEK that were pretreated with CAPE as were observed for cells post-treated with CAPE 30 min after exposure to SM or NM (Fig. 4, A and B, Post: Vehicle versus Post: CAPE). These data suggest that CAPE alters the fate of cells exposed to alkylating agent. However, CAPE itself induces cell death by 24 h in NHEK (Fig. 4B, Cont, Pre: CAPE) and by 48 h in the p53-mutated HaCaT cells (not shown). These data are consistent with recent findings that CAPE might induce apoptosis via mechanisms both dependent and independent of p53 transcriptional activity.

**BFA Induce Phosphorylation of p53.** Exposure to DNA-damaging agents can activate nonclassical NF- $\kappa B$  activity that is mediated by transcription-independent mechanisms of p53 (Bohuslav et al., 2004). A p53 response is typically characterized by an increase in total p53 protein levels several hours after a DNA-damaging event (Rosenthal et al., 1998). However,



**Fig. 2.** CAPE inhibits classical NF- $\kappa B$ -mediated reporter gene activity. Keratinocyte reporter gene HaCaT.AL cells were pretreated for 1.5 h with vehicle (DMSO) or 105  $\mu M$  CAPE and exposed to media alone (control) or 0.5 ng/ml TNF $\alpha$  for 4, 6, 9, 12, or 20 h. Cell lysate was collected and measured for luciferase reporter gene activity. The luciferase activity value for TNF $\alpha$ -exposed cells was divided by the luciferase activity value of the corresponding media-treated control cell time point. Data were further normalized to LDH activity measured in cell medium to account for damaged/nonviable cells (normalized luciferase activity). The control value is denoted as the value of 1. These data are representative of three experiments. Error bars represent S.E.M. Asterisks (\*) indicate statistically significant decreases in reporter gene activity by treatment with CAPE (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ). Daggers (†) indicate statistically significant increases in reporter gene activity by treatment with TNF $\alpha$  (†,  $p < 0.05$ , ††,  $p < 0.01$ ).





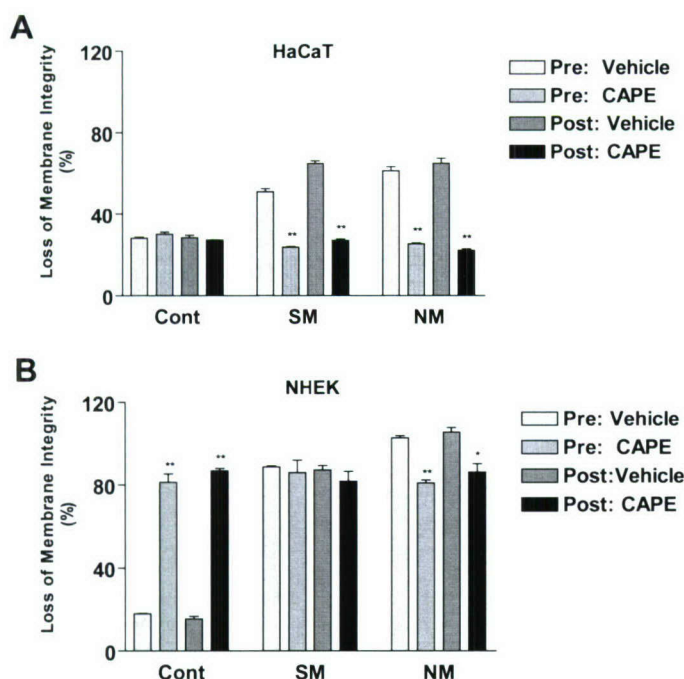
**Fig. 3.** CAPE inhibits nonclassical NF- $\kappa$ B-mediated reporter gene activity. A, keratinocyte reporter gene HaCaT.AL cells were exposed to media alone (untreated control luciferase activity is equal to 1 at each time point) or 12.5, 50, 100, or 200  $\mu$ M SM for 4, 6, 9, 12, or 20 h. Cell lysate was collected and measured for luciferase reporter gene activity. Data were further normalized to LDH activity measured in cell medium to account for damaged/nonviable cells (normalized luciferase activity). These data are representative of five independent experiments. Cells were pretreated with vehicle (DMSO) or 105  $\mu$ M CAPE for 1.5 h and then exposed to 12.5  $\mu$ M (B), 50  $\mu$ M (C), 100  $\mu$ M (D), or 200  $\mu$ M (E) SM for 4, 6, 9, 12, or 20 h. Cell lysate was collected and measured for luciferase reporter gene activity to determine whether CAPE could inhibit SM-mediated reporter gene activity. Data were further normalized to LDH activity measured in cell medium to account for damaged/nonviable cells (normalized luciferase activity). The DMSO-pretreated, unexposed control luciferase activity is equal to the value of 1 at each time point. These data are representative of three experiments. Error bars represent S.E.M. Asterisks (\*) indicate statistically significant decreases in reporter gene activity by treatment with CAPE (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ) in B, C, D, and E. Daggers (†) indicate statistically significant increases in reporter gene activity induced by treatment with SM (†,  $p < 0.05$ ; ††,  $p < 0.01$ ) in B, C, D, and E.

an increase in total p53 protein levels is difficult to observe in HaCaT cells (Fig. 5A, bottom). Total p53 protein levels increase slightly by 4 h in NHEK exposed to 200  $\mu$ M SM (Fig. 5B, bottom). Monitoring phosphorylation of p53 can serve as a more rapid indicator of a p53 response. Phosphorylated p53 accumulates in both HaCaT cells (Fig. 5, A and C) and NHEK (Fig. 5, B and D) within 15 to 30 min after exposure to 200  $\mu$ M SM. Both cell lines exhibit a time- and concentration-dependent increase in phosphorylated p53. The accumulation of phosphorylated p53 following 200  $\mu$ M NM exposure temporally lags behind that induced by 200  $\mu$ M SM in both cell types (Fig. 5, C and D). Exposure of cells to CEES or TDG does not result in accumulation of phosphorylated p53 (Fig. 5, C and D). The positive control chemical HU induces accumulation of phosphorylated p53 in both cell types (Fig. 5, C and D). These data suggest that exposure of both NHEK and HaCaT cells to BFA can initiate signaling involved in directing the initial events of the p53 response.

**CAPE Inhibits Alkylating Agent-Induced Phosphorylation of p53.** We examined whether pretreatment (1.5 h before exposure) or post-treatment (30 min after exposure) of cells with CAPE modulated alkylating agent-induced phosphorylation of p53. Treatment of NHEK or HaCaT cells with CAPE alone for 7.5 h did not result in phosphorylation of p53 (not shown). Pretreatment and post-treatment of cells with CAPE either inhibited or reduced phosphorylation of p53 in NHEK and HaCaT cells, respectively (Fig. 6A). The moderate accumulation of p53 observed by 4 h after alkylating agent exposure was also inhibited by pretreatment and post-treatment with CAPE (Fig. 6B). These data suggest that CAPE modulates alkylating agent-induced phosphorylation of p53 and may potentially disrupt p53-related activation of non-classical NF- $\kappa$ B signaling.

**CAPE Inhibits Alkylating Agent-Induced Phosphorylation of p90RSK.** p90RSK is thought to play a role in non-classical NF- $\kappa$ B activation. p90RSK accumulates in the nucleus





**Fig. 4.** CAPE decreases the loss of cell membrane integrity. HaCaT keratinocytes (A) and NHEK (B) were pretreated (for 1.5 h pre-exposure) or post-treated (30 min postexposure) with DMSO (vehicle) or 105  $\mu$ M CAPE (CAPE) and either unexposed (Cont) or exposed to 200  $\mu$ M SM or NM for 24 h. Cell medium was collected and analyzed for LDH activity, a measure of membrane integrity and a correlate of cell viability, as described under *Materials and Methods*. To establish the maximal LDH activity achievable from these cells, untreated control cells were grown for 24 h and then lysed using detergent (expressed as 100% loss of membrane integrity). These data are representative of three experiments. Error bars represent S.E.M. Asterisks (\*) indicate statistical significant difference as a result of CAPE treatment compared with its corresponding vehicle control (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ).

as a result of physical association with p53 following exposure to DNA-damaging agents (Bohuslav et al., 2004). p90RSK can activate NF- $\kappa$ B by phosphorylating I $\kappa$ B and/or the NF- $\kappa$ B subunit p65 (Ghoda et al., 1997; Schouten et al., 1997; Bohuslav et al., 2004). Therefore, we examined whether CAPE could modulate p90RSK-related signaling. Cells were pretreated or post-treated with CAPE and exposed to SM or NM. Exposure of NHEK to SM or NM alone resulted in accumulation of phosphorylated p90RSK between 1 and 4 h after exposure (Fig. 7A, top two panels). CAPE pretreatment of NHEK inhibited alkylating agent-induced phosphorylation of p90RSK (Fig. 7A). HaCaT cells treated with CAPE alone or CAPE plus alkylating agent resulted in accumulation of phosphorylated p90RSK only at the 1 h time point (Fig. 7A, bottom two panels). That is, in HaCaT cells, CAPE treatment together with alkylating agent exposure resulted in increased accumulation of phosphorylated p90RSK. Total p90RSK protein levels remained unchanged (Fig. 7B, solid arrows), but exposure to NM resulted in accumulation of high molecular weight protein complexes recognized by the p90RSK antibody, particularly in HaCaT cells (Fig. 7B, dashed arrows). These data suggest that alkylating agents activate p90RSK and that CAPE might disrupt p90RSK signaling.

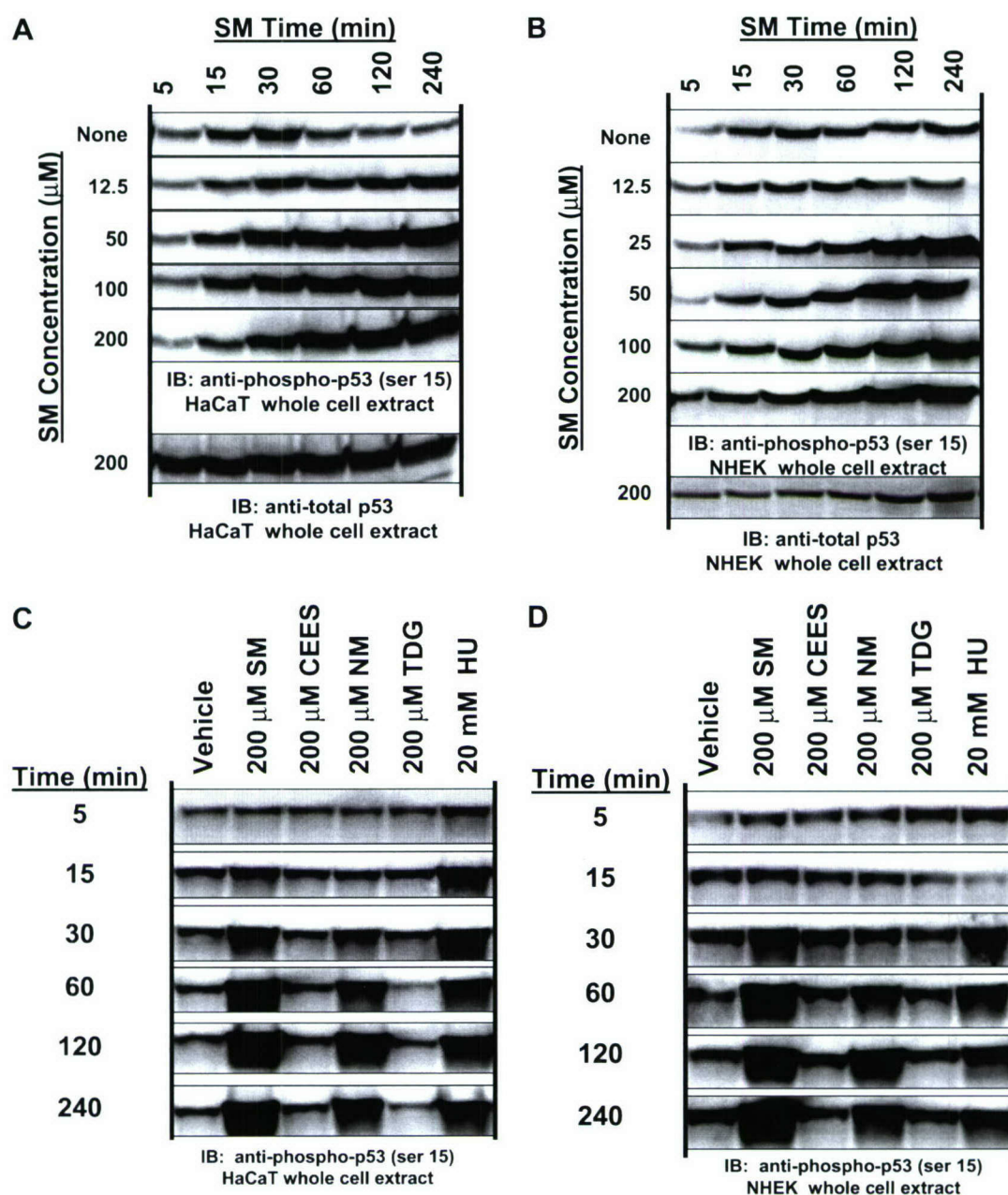
**CAPE Treatment and Alkylating Agent Exposure Modulate Nrf2 Protein Expression.** The transcription factor Nrf2 mediates ARE/EpRE signaling, affects p53 stabilization (Vasiliou et al., 2003), and modulates expression of

NF- $\kappa$ B family members (Yang et al., 2005). Nrf2 protein levels increase in various systems when exposed to chemopreventive compounds (Chen and Kong, 2004). We examined whether Nrf2 protein expression was altered in NHEK or HaCaT cells following treatment with CAPE and/or exposure to alkylating agents. Exposure of NHEK to NM resulted in a slight accumulation of an  $\sim$ 110 kDa Nrf2 at 1, 4, and 6 h after exposure (Fig. 8A, solid arrows). However, treatment of NHEK with CAPE plus alkylating agent (particularly NM) resulted in an accumulation of high molecular weight protein complexes recognized by the anti-Nrf2 antibody (Fig. 8A, dashed arrows) ( $\sim$ 150–200 kDa). In NHEK and HaCaT cells, 105  $\mu$ M CAPE by itself did not result in accumulation of 110 kDa Nrf2 forms (Fig. 8), even following 7.5 h of treatment (not shown). That is, multiple molecular weight forms of Nrf2 protein are observable following treatment with CAPE and exposure to alkylating agents in keratinocytes. These data suggest that alkylating agents in combination with CAPE might be able to modify the Nrf2 signaling complex that is involved in initiating ARE/EpRE signaling.

**CAPE Treatment Modulates Steady-State AhR Protein Expression.** It has recently been discovered that Nrf2 gene transcription is directly modulated by the AhR (Miao et al., 2005). We investigated whether CAPE could alter an AhR response because a structurally similar compound, curcumin, has been shown to modulate AhR activity (Ciolino et al., 1998). Binding of the prototypical ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the AhR results in loss of AhR within 5 h in Hepa1c1c7 cells (Ma and Baldwin, 2000). NHEK and HaCaT cells were pretreated or post-treated with CAPE and exposed to SM or NM (Fig. 8B). Interestingly, exposure of NHEK and particularly of HaCaT cells to NM alone, but not SM, resulted in a decrease of detectable AhR protein (Fig. 8B, solid arrows) ( $\sim$ 105 kDa), which were further decreased by treatment with CAPE. After 7.5 h of treatment with CAPE alone, both NHEK and HaCaT cells exhibited a loss of AhR protein (Fig. 8C). Exposure to NM with and without CAPE resulted in accumulation of a high molecular weight protein complex recognized by the anti-AhR antibody, particularly from NHEK cell lysates (Fig. 8B, dashed arrows) ( $>$ 200 kDa). These data suggest that the AhR may play a role in the cellular response to BFA and in activation of ARE/EpRE signaling following treatment with CAPE.

**CAPE Decreases Alkylating Agent-Induced Necrotic-Like Morphology but Induces Cell Blebbing and Half-Body Formation.** CAPE can modulate keratinocyte signaling and prevent overt loss of cell membrane integrity caused by alkylating agents (Figs. 1–8). However, CAPE itself has been shown to be cytotoxic and to induce apoptosis. Pretreatment (Fig. 9) or post-treatment (not shown) of HaCaT cells and NHEK with CAPE reduced alkylating agent-induced cell detachment and necrotic-like morphology (compare Fig. 9Ab with Af and Fig. 9Ac with Ag). However, close examination reveals CAPE-induced cell blebbing by 9 h in HaCaT cells (Fig. 9Ah, 1) and half-body formation after 24 h in NHEK cells (Fig. 9Bh, 2). These data suggest that CAPE might change cell fate from alkylating agent-induced necrotic-like cell death signaling to that of an independent pathway of cell death (i.e., programmed cell death).





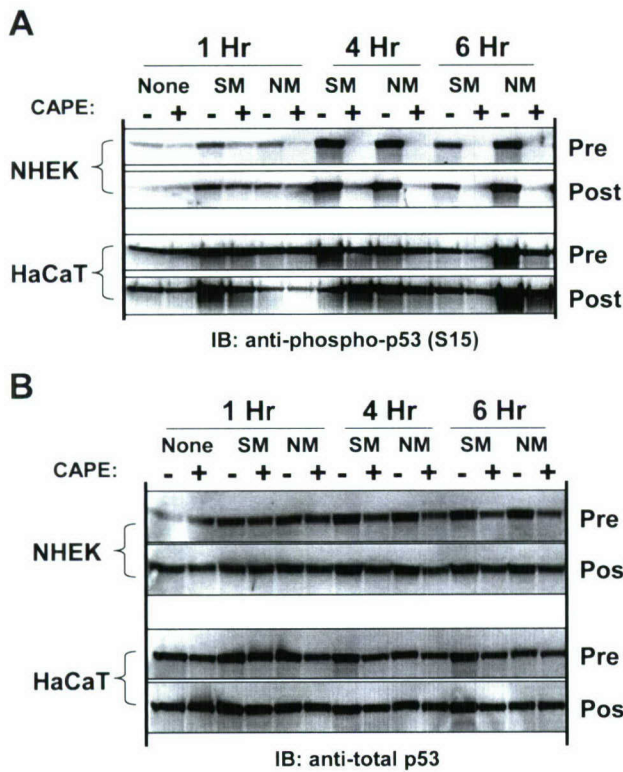
**Fig. 5.** BFA rapidly induce phosphorylation of p53 at residue serine 15. HaCaT keratinocytes (A) and NHEK (B) were exposed to 12.5, 25, 50, 100, or 200  $\mu\text{M}$  SM for 5, 15, 30, 60, 120, or 240 min. Whole-cell extracts were isolated and IB using specific anti-phospho-p53 (Ser15) antibodies (top panels for each) or anti-p53 (total p53) antibodies (bottom panel for each) following separation by SDS-PAGE. HaCaT keratinocytes (C) and NHEK (D) were exposed to vehicle (0.02% DMSO), 200  $\mu\text{M}$  SM, CEES, NM, TDG, or 20 mM HU (positive control) for 5, 15, 30, 60, 120, or 240 min. Whole-cell extracts were isolated and IB using specific anti-phospho-p53 (Ser15) antibodies following separation by SDS-PAGE. These data are representative of two independent experiments for each treatment.

## Discussion

The precise mechanisms that govern NF- $\kappa$ B and p53 responses to BFA are not completely understood. BFA are used as therapeutics in cutaneous oncology and have been used as chemical warfare agents. Understanding the molecular mechanisms of BFA may help identify efficacious chemoprotectants/therapeutics to ameliorate BFA-induced cutaneous intolerance, inflammatory responses, blister formation, and cancerous or precancerous lesions. Because p53 and NF- $\kappa$ B signaling are modulated by BFA and because the balance between NF- $\kappa$ B and p53 signaling can determine toxic effects and cell fate, we addressed the BFA-induced responses of

these critical pathways in skin cells. We showed that BFA, not monofunctional agents, can induce phosphorylation of p53 before initiating NF- $\kappa$ B signaling, even in cells that express a transcriptionally inactive p53 protein. These data support and extend previous findings suggesting that DNA-damaging agents alter p53 function, which in turn is required for activation of nonclassical NF- $\kappa$ B signaling mediated by p90RSK. The nontraditional NF- $\kappa$ B inhibitor CAPE disrupts p53 and p90RSK signaling and subsequently inhibits NF- $\kappa$ B activity. These data suggest that it is not appropriate to designate CAPE as a specific NF- $\kappa$ B inhibitor. Furthermore, CAPE modifies ARE/EpRE signaling pathways

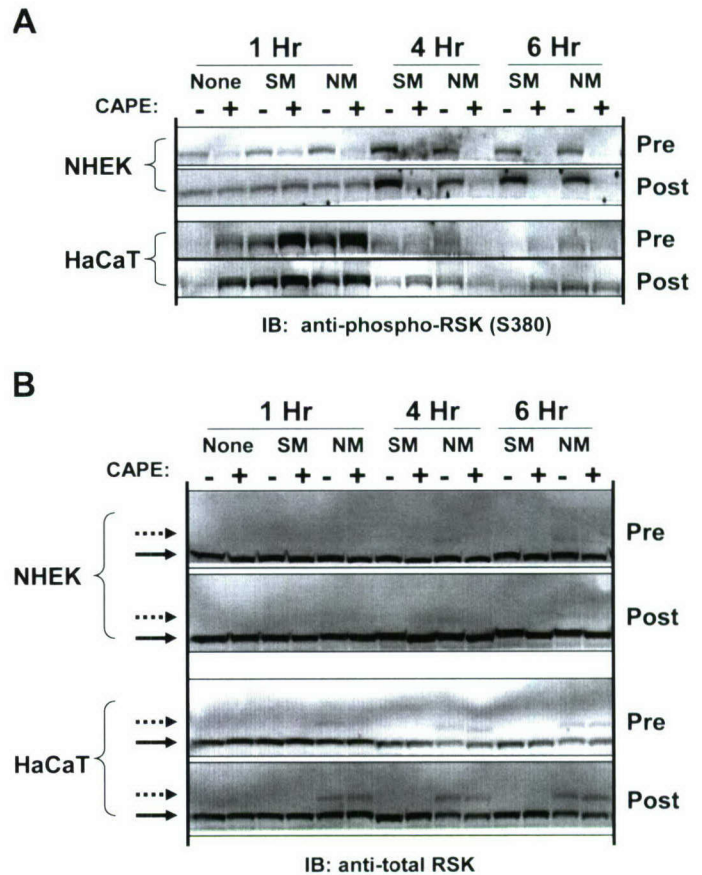




**Fig. 6.** CAPE inhibits alkylating agent-induced phosphorylation of p53. HaCaT keratinocytes and NHEK were pretreated (for 1.5 h) or post-treated (30 min postexposure) with vehicle (–) or 105  $\mu$ M CAPE (+) and either unexposed (None) or exposed to 200  $\mu$ M SM or NM for 1, 4, or 6 h. Whole-cell extracts were isolated and IB using specific anti-phospho-p53 (Ser15) antibodies (A) or anti-total p53 antibodies (B) following separation by SDS-PAGE. These data are representative of two independent experiments for each post-treatment and pretreatment. In NHEK pretreated with CAPE and then exposed to SM or NM, CAPE inhibited p53 phosphorylation ~2-fold in SM-exposed cells at 1 h, ~5-fold in both SM- and NM-exposed cells at 4 h, and ~3-fold in SM-exposed cells or ~2-fold in NM-exposed cells at 6 h. In NHEK post-treated with CAPE following exposure to SM or NM, CAPE inhibited p53 phosphorylation ~2-fold at 1 h, ~3-fold in SM-exposed cells or ~4-fold in NM-exposed cells at 4 h, and ~2-fold in SM-exposed cells or ~6-fold in NM-exposed cells at 6 h. In HaCaT keratinocytes pretreated with CAPE and then exposed to SM or NM, CAPE inhibited p53 phosphorylation ~2-fold in NM-exposed cells at 4 h and ~4-fold in NM exposed cells at 6 h. In HaCaT keratinocytes post-treated with CAPE following exposure to SM or NM, CAPE inhibited p53 phosphorylation ~3-fold in SM-exposed cells at 1 h and ~2-fold in NM-exposed cells at 6 h.

that are modulated in response to BFA-induced stress. The timing and degree of modulation of the p53, NF- $\kappa$ B, p90RSK, and ARE/EpRE pathways by BFA and/or CAPE show differences in immortalized keratinocytes versus normal keratinocytes. Therefore, intrinsic differences between normal and immortalized skin cells will determine the outcome of exposure to BFA or treatment with chemoprotectants.

Transcription-independent mechanisms of p53 protein have been suggested to play a role in activating nonclassical NF- $\kappa$ B signaling initiated by DNA-damaging agents (Bohuslav et al., 2004; Ryan et al., 2004). Bohuslav et al. (2004) suggested that the classical IKK/I $\kappa$ B signaling pathway is not involved in p53-mediated NF- $\kappa$ B activity. In doxycycline-activated Saos-2 p53-inducible cells, phosphorylation and degradation of I $\kappa$ B $\alpha$  were not necessary to observe relocalization of the NF- $\kappa$ B subunit p65 to the nuclear compartment. Also, an NF- $\kappa$ B reporter gene was activated by over-

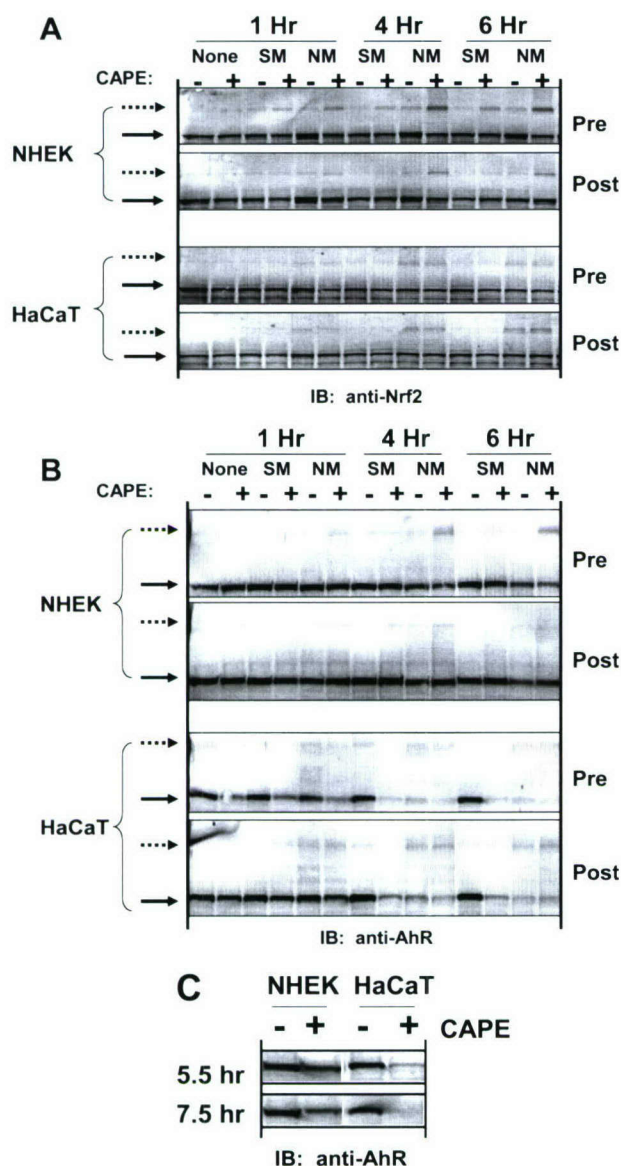


**Fig. 7.** CAPE inhibits alkylating agent-induced phosphorylation of p90RSK. HaCaT keratinocytes and NHEK were pretreated (for 1.5 h) or post-treated (30 min postexposure) with vehicle (–) or 105  $\mu$ M CAPE (+) and either unexposed (None) or exposed to 200  $\mu$ M SM or NM for 1, 4, or 6 h. Whole-cell extracts were isolated and IB using specific anti-phospho-RSK (ser 380) antibodies (A) or anti-total RSK antibodies (B) following separation by SDS-PAGE. Solid arrows indicate the 90 kDa form of RSK, whereas dashed arrows indicate a high molecular weight form of RSK. These data are representative of two independent experiments for each post-treatment and pretreatment.

expression of p53 in this system, even though overexpression of p53 did not activate IKK. Furthermore, NF- $\kappa$ B reporter gene activation occurred in p53-overexpressing, IKK1/2-deficient murine embryonic fibroblasts. These data together suggest that p53-mediated nonclassical NF- $\kappa$ B activation may be a critical pathway involved in inducing the toxic effects of BFA.

The data presented here are consistent with the observations that BFA-induced NF- $\kappa$ B activity is independent of the transcriptional activity of p53 (Bohuslav et al., 2004). Immortalized HaCaT cells, which express a full-length but transcriptionally inactive p53 protein, were compared with NHEK to determine whether p53 could be phosphorylated and to investigate whether BFA could induce I $\kappa$ B $\alpha$  degradation and/or activate an NF- $\kappa$ B-responsive reporter gene. Both HaCaT cells and NHEK responded to BFA exposure by initiating phosphorylation of p53 (Fig. 5). Observable loss of I $\kappa$ B $\alpha$  did not occur until 2 h of exposure to 200  $\mu$ M SM in NHEK or until 6 h in HaCaT cells (Fig. 1B). BFA-induced transcriptional activity of an NF- $\kappa$ B reporter gene was not observed until 6 to 9 h in the stably transfected HaCaT cells (Fig. 3). That is, BFA induce phosphorylation of p53, a known

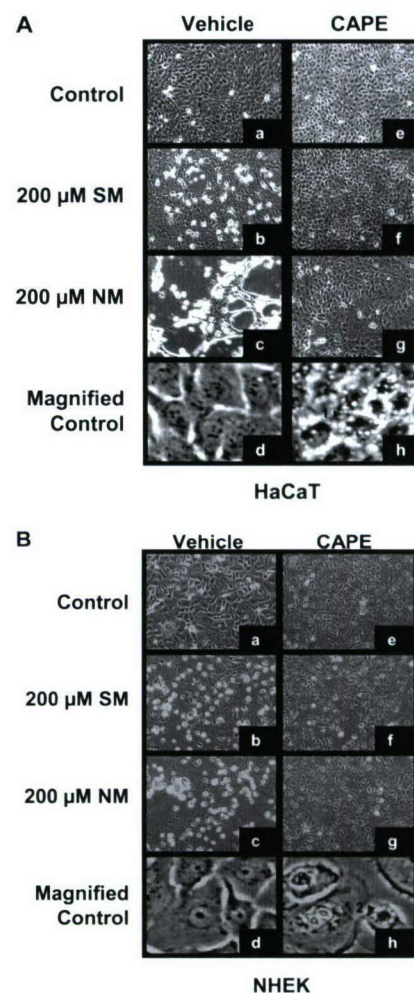




**Fig. 8.** CAPE treatment and alkylating agent exposure modulates Nrf2 and AhR protein expression patterns. HaCaT keratinocytes and NHEK were pretreated (for 1.5 h) or post-treated (30 min postexposure) with vehicle (-) or 105  $\mu$ M CAPE (+) and either unexposed (None) or exposed to 200  $\mu$ M SM or NM for 1, 4, or 6 h. Whole-cell extracts were isolated and IB using specific anti-Nrf2 antibodies (A, solid arrows indicate the ~110 kDa Nrf2, whereas dashed arrows indicate a high molecular weight form of Nrf2) or anti-AhR antibodies (B, solid arrows indicate the ~105 kDa AhR, whereas dashed arrows indicate a high molecular weight form of AhR) following separation by SDS-PAGE. C, HaCaT keratinocytes and NHEK were treated with vehicle (-) or 105  $\mu$ M CAPE (+) for 5.5 or 7.5 h. Whole-cell extracts were isolated and IB using anti-AhR antibodies following separation by SDS-PAGE. These data are representative of two independent experiments for each post-treatment and pretreatment.

early step in the p53 response, and subsequently initiate I $\kappa$ B $\alpha$  degradation and NF- $\kappa$ B reporter gene activation, both indicators of a BFA-induced NF- $\kappa$ B response.

How can the p53 protein modulate BFA-induced NF- $\kappa$ B activity independent of p53-mediated transcription? Doxorubicin-induced DNA damage activates the kinase ATM, which in turn activates a mitogen-activated protein kinase kinase (MEK)/extracellular signal-activated kinase (ERK)/p90RSK signaling cascade (Panta et al., 2004). Although p53 is a



**Fig. 9.** CAPE decreases alkylating agent-induced necrotic-like morphology but induces cell blebbing and half-body formation. HaCaT keratinocytes and NHEK were pretreated (for 1.5 h) with vehicle (a-d) or 105  $\mu$ M CAPE (e-h) and either unexposed (Control; a and e) or exposed to 200  $\mu$ M SM (b and f) or NM (c and g) for 9 h in HaCaT cells (A) or 24 h in NHEK (B). Cell images were visualized with a 10 $\times$  eyepiece and 10 $\times$  objective and acquired using a digital camera system. The Magnified Control (d and h) is an ~8 $\times$  digital magnification of Control cells pretreated with vehicle (a) or CAPE (e) to emphasize that CAPE by itself induces morphological changes such as cell blebbing (Ah) and half-body formation (Bh). These data are representative images from three independent experiments.

substrate for ATM, this DNA damage-mediated cascade can occur independently of the p53 protein. p90RSK can also become activated following shuttling into the nucleus, via physical association with p53, after exposure to DNA-damaging agents (Bohuslav et al., 2004). Transcriptionally inactive p53 proteins still associate with p90RSK (Bohuslav et al., 2004), translocate into the nucleus where p90RSK can phosphorylate p65 (Bohuslav et al., 2004) and/or I $\kappa$ B $\alpha$  (Ghoda et al., 1997; Schouten et al., 1997), and thereby allow activation of the normally shuttling NF- $\kappa$ B complex. We show that SM or NM exposure induces phosphorylation of p90RSK in both NHEK and HaCaT cells, although with different temporal regulation (Fig. 7). Because p90RSK can be phosphorylated by MEK/ERK and/or autophosphorylated (Dalby et al., 1998), these data suggest that BFA activate the MEK/ERK/p90RSK pathway. BFA stimulate phosphorylation and stabilization of p53, activation of p90RSK, and



subsequent activation of NF- $\kappa$ B, further delineating the mechanism of action of BFA-induced nonclassical NF- $\kappa$ B signaling.

CAPE is an active phenolic constituent found in propolis. CAPE, which has been described as a specific inhibitor of NF- $\kappa$ B (Natarajan et al., 1996), was used to further dissect BFA-induced nonclassical NF- $\kappa$ B signaling. CAPE has been shown to inhibit NF- $\kappa$ B translocation into the nucleus via a mechanism that apparently does not depend on I $\kappa$ B $\alpha$  stabilization or inhibition of IKK (Natarajan et al., 1996). CAPE inhibited phosphorylation and accumulation of p53 (Fig. 6) and phosphorylation of p90RSK (Fig. 7). In HaCaT cells, treatment with CAPE alone resulted in phosphorylation of p90RSK, whereas CAPE in combination with an alkylating agent resulted in an additive effect on the accumulation of phosphorylated p90RSK within 1 h of exposure to the alkylating agent (Fig. 7). The differences between the p90RSK response in NHEK and HaCaT cells may be related to the p53 status of the cells. HaCaT cells exhibit a p53 mutational spectrum commonly attributed to UV light exposure that translates into a transcriptionally inactive p53 protein (Lehman et al., 1993). Overall, these data suggest that CAPE might interfere with nonclassical NF- $\kappa$ B activation by disrupting BFA-induced p53 and MEK/ERK/p90RSK signaling.

Some protective effects of phenolic compounds such as CAPE or curcumin may be attributed to their ability to activate ARE/EpRE signaling (Chen and Kong, 2004). It has been shown that CAPE or curcumin activates the transcription factor Nrf2 (Balogun et al., 2003). Nrf2 is a principal regulator of ARE/EpRE-mediated transactivation involved in inducing expression of phase II detoxification and antioxidant enzymes (Lee and Surh, 2005). Nrf2 activation is involved in protection against chemical-induced damage and oxidative stress (Lee and Johnson, 2004). Even post-treatment (30 min after BFA exposure) with CAPE protected cells from BFA-induced membrane integrity loss (Fig. 4). This suggests a rapid mechanism by which CAPE decreases the toxic effects of BFA.

ARE/EpRE and Nrf2 signaling have been shown to be modulated by AhR (Miao et al., 2005). Curcumin has been shown to compete with dioxin, the canonical ligand of AhR, for binding to AhR (Ciolino et al., 1998). Curcumin stimulates AhR to interact with xenobiotic response elements (XRE). The Nrf2 promoter contains several XRE-like elements to which the AhR directly binds (Miao et al., 2005). Exposure of Hepa1c1c7 cells to dioxin results in ubiquitin-proteasomal degradation of the AhR within 5 h (Ma and Baldwin, 2000). Interestingly, exposure of HaCaT cells to NM alone, but not SM alone, resulted in loss of steady-state levels of the AhR (Fig. 8). CAPE treatment alone resulted in reduced AhR protein levels in NHEK and in nearly complete loss of steady-state AhR levels within 5.5 h in HaCaT cells (Fig. 8C). Furthermore, treatment with CAPE in combination with exposure to NM in NHEK results in high molecular weight protein complexes containing the AhR (>200 kDa) (Fig. 8B) as it did for both p90RSK (~100–150 kDa) (Fig. 7B) and Nrf2 (~150–200 kDa) (Fig. 8A). The differences between NHEK and HaCaT cells may be related to the observation that the AhR compartmentalization is dependent on cell density in HaCaT cells (Ikuta et al., 2004). This quite novel regulation of AhR is thought to be regulated by phosphorylation/dephosphorylation events (Ikuta et al., 2004). CAPE

may modulate kinase/phosphatase activity responsible for this novel AhR relocalization in HaCaT cells.

Typical AhR modulators cause a conformational change to AhR and its associated chaperone protein complex, 90-kDa heat shock protein/XAP2 (hepatitis B virus X-associated protein 2)/p23, that allows AhR to translocate to the nucleus, associate with its dimerization partner AhR nuclear translocator, and activate XRE-dependent gene transcription. Treatment with CAPE may similarly alter the AhR-chaperone protein complex such that previously unexposed residues and/or other AhR-interacting proteins can be cross-linked by NM. The high molecular weight protein complexes for p90RSK, Nrf2, and AhR may contain a common protein target of CAPE and/or BFA, such as a chaperone or associating protein(s). These high molecular weight complexes may be induced by cross-linking caused by BFA. We have shown previously that SM has the capacity to cross-link biological molecules in cells (Dillman et al., 2003). We do not expect these results are caused by nonspecific antibody reactions because the high molecular weight species consistently appear only in certain exposure groups. Furthermore, we speculate that CAPE, like curcumin, can modulate AhR signaling by either directly interacting with the AhR ligand binding domain and/or disruption of AhR-chaperone complexes and/or phosphorylation/dephosphorylation signaling required for AhR localization and activity.

CAPE has been shown to induce apoptosis in multiple cell types. Here we show that CAPE prevented cell detachment and necrotic-like cell viability loss caused by BFA. However, it is important to note that although human immortalized HaCaT keratinocytes did not exhibit membrane integrity loss within 24 h after treatment with CAPE alone or with CAPE in combination with BFA (Fig. 9A), they did after 48 h (not shown). NHEK exhibited membrane integrity loss after 24 h of exposure to CAPE (Fig. 9B). It appears that CAPE can "hijack" cell machinery to prevent cell detachment and necrotic-like death induced by 200  $\mu$ M concentrations of SM or NM. However, it also directs cells to undergo cellular blebbing and half-body formation, consistent with its capacity to induce apoptosis. CAPE may be involved in anoikis, that is, apoptosis induced by the loss of integrin-mediated cell-matrix contact (Frisch and Ruoslahti, 1997), because subapoptotic concentrations of CAPE caused rearrangement of the cytoskeleton and reduced tyrosine phosphorylation of focal adhesion kinase (Weyant et al., 2000). Also, CAPE-induced signaling resulting in cell blebbing and half-body formation may be indicative of the cellular defense mechanism, autophagy, similar to that observed of other dietary chemopreventive agents (Ellington et al., 2006; Herman-Antosiewicz et al., 2006). Thus, CAPE seems to prevent BFA-induced toxicity by modulating not only NF- $\kappa$ B signaling but also p53, p90RSK, and ARE/EpRE signaling while it concurrently drives cell matrix changes and programmed cell death.

In summary, we show that BFA such as SM and NM can induce nonclassical NF- $\kappa$ B activation, rapidly induce phosphorylation of p53 in both immortalized and normal skin cells, modulate p90RSK activation, and activate ARE/EpRE signaling. Also, exposure to NM caused accumulation of high molecular weight proteins for p90RSK, Nrf2, and AhR. Exposure of keratinocytes to NM alone, but not SM, resulted in altered Nrf2 protein expression and loss of steady-state AhR protein. The mechanisms governing these novel observations



will be pursued, and we hypothesize that they may be related to differences in reactivity of different BFA with biomolecules and/or mechanisms of cellular transport into cells. For example, NM is transported into cells via choline transporters (Goldenberg et al., 1971), whereas SM is lipophilic and crosses cell membranes readily (Papirmeister et al., 1991). Differences in NM-mediated and SM-mediated effects on cellular systems have been observed and commented on by other investigators (e.g., Rappeneau et al., 2000). The ability of CAPE to alter keratinocyte fate following exposure to BFA is characterized by inhibition of p53, p90RSK, and NF- $\kappa$ B signaling and modulation of ARE/EpRE pathways. CAPE may be used to further define the molecular mechanisms of BFA-induced nonclassical NF- $\kappa$ B signaling pathways and to ameliorate subsequent toxic effects.

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